Comparison of bacterial biofilm communities using barcoded pyrosequencing and analysis to determine origin of biofilm fouling of reverse osmosis membranes in a full scale desalination system.

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Abstract
Biofouling is the single most important issue in reverse osmosis sea water desalination worldwide (Ridgway et al., 1999) and may account for up to 50% of energy use. Which species are responsible and their origin is unclear. With the advent of next generation sequencing, species diversity and transience can be examined at orders of magnitude greater detail than was previously possible. We found many similarities in bacterial families across source water, prefiltration units and membranes in this study and in the few other studies available, despite disparate locations and seasons. Key groups included members of the Bacteroidetes (e. *Flavobacteriaceae*), Planctomycetes, Alphaproteobacteria (eg. *Rhodobacteraceae*, *Sphingomonadales*), Betaproteobacteria (eg. *Burkholderia*) and Gammaproteobacteria (eg. *Oceanospirillales*, *Xanthomonadaceae*). Despite similarities in families, the predominant fouling species on reverse osmosis (RO) membranes appear to differ between studies. This seems likely to reflect a common origin (seawater) but subsequent adaptation or selective pressures in different niches, particularly on RO membranes under high pressure and salt concentration. We can now select environmental isolates from our culture collection representing key bacterial groups responsible for biofouling in seawater systems. This will enable more accurate evaluation of the effectiveness of anti-fouling strategies.

Keywords
16S rRNA; community profling; pyrosequencing; SWRO; biofouling; RO membrane.

Introduction
The requirement for fresh drinking water continues to increase worldwide and seawater reverse osmosis (SWRO) plants are becoming an increasingly popular solution with many new plants constructed over the last few years, with two in Western Australia. Membrane biofouling is the single most important issue in reverse osmosis sea water desalination (Ridgway et al., 1999). The economics of biofouling control is important in the long term energy use and greenhouse gas emission of desalination plants. Energy costs and associated greenhouse gas emissions could be reduced by up to 50% if fouling on membranes could be measured and controlled. It is unclear, however, which species to monitor and where to sample.

Pretreatment steps vary among different plants but most have some form of large sieving followed by chemical treatments to increase flocculation and coagulation, then prefiltration stages which may include one or more of the following: sand/media filtration; microfiltration to remove particles of >10µM; ultrafiltration to remove particles between 1-10 µM. Many colloids and particulates are removed during pre-treatment but some bacteria appear to traverse even ultrafiltration units (Chun et al. 2012). This may result from small regions of filter damage during manufacture or use or result from bacteria of smaller sizes (eg. *Microbacteriaceae*) or flexibility (eg. viable but not culturables – VBNC) or perhaps non-sterile conditions at start-up. These types of bacteria are commonly found in oligotrophic marine environments.

A range of biological materials can contribute to the fouling of reverse osmosis membranes. During analyses of reverse osmosis membranes in a full scale plant over their 6-year lifespan, Armstrong et al. (2009) estimated biofilms and organic fouling accounted for 49% of the total fouling. Bacteria in biofilms cause biofouling largely through the production of copious amounts of sticky extracellular polysaccharides (EPS), representing up to 95% of their biomass (Sutherland, 1990; Skillman et al., 1999). To understand the relative contribution of source water and biofilms to organic membrane fouling, we need more information about the bacterial species present and the types of polymers they produce.

Most membrane biofouling research to date has focussed on freshwater and wastewater treatment units, with only a handful of studies investigating seawater systems. Although there is some information on marine biofilms, high pressure seawater environments characterising seawater reverse osmosis (SWRO) membranes remain largely unexplored (Manes et al., 2011). In part this is due to the difficulties in sampling the sealed spiral wound membrane units which are expensive and have a typical lifespan of 5 years. It is therefore of interest to investigate if other locations with easier access or more frequent replacement could be used to indicate membrane fouling. Of the few studies available, there is conflicting evidence for dominant fouling species with dynamic population changes over time.

While the dominant Sanger sequencing method has been used for over 30 years, new high throughput sequencing technologies are causing a fundamental shift in molecular biology. These new methods are referred to as next generation sequencing (NGS), including systems such as Roche/454, Illumina/Solexa, Applied Biosystems/SOLiD, and Helicos BioSciences. These methods enable rapid characterization of targeted sequences and cost much less than traditional Sanger sequencing (Metzker, 2009). NGS has the potential to provide new insight into the entire genome of the seawater environment, including genes that are present within the microbial community at very low levels.

The predominant bacterial groups in the biofilms identified in seawater systems vary between studies. Many indicate the prevalence of alphaproteobacteria in seawater (Joint et al., 2010) and RO membranes (Lee et al., 2009; Manes et al., 2011) including species of the *Rhodobacteriaceae* family (Sibani et al., 2007). Within the *Rhodobacteriaceae* family, *Roseobacter* and *Sphingomonas* spp. have been suggested as primary colonisers in several studies (Dang and Lovell, 2000; Bereschenko et al., 2010; Lee et al., 2009). In a recent study, Chun et al. (2012) compared bacterial communities from cartridge filters and RO membranes using conventional Sanger sequencing. They found higher proportions of *Bacillus* spp. in prefilters and a larger proportion of Bacteroidetes, Planctomycetes and Chloroflexi on RO membranes.

We were interested in comparing bacterial communities from a full scale desalination system in Western Australia to determine similarities in other locations around the world with the aim of identifying suitable model bacterial groups for further experiments. There are significant gaps in knowledge of fouling in desalination processes. Many studies use unsuitable model bacteria for fouling tests such as fresh water isolates (*Escherichia coli*). Whilst it is possible to access the genetic information in uncultured organisms through genomics (Glöckner and Joint, 2010), it is clear that the potential of any organism can best be achieved by having that particular organism available for experimentation in the laboratory. Even in this age of high-throughput DNA sequencing, cultures are still essential. They provide almost the only way to discover the physiology of microbes, to establish which organic substrates are used, to determine what secondary metabolites might be released, or biotransformations might be possible (Joint et al, 2010).

Once problematic biofilm species are identified and key isolates obtained, more accurate models can be used to evaluate the effectiveness of anti-fouling strategies.

Methods

Process Overview

The Perth Seawater Desalination Plant (PSDP) was the first of Australia's plants to provide desalinated seawater for large scale public consumption. It was completed in 2006 and produces up to 45 gigalitres of fresh drinking water per year. It is located on the coast, around 25km South of the City of Perth in Western Australia. The Seawater intake pipe is around 1km in length and collects water from Cockburn Sound. We have collected samples from different locations at PSDP, across seasons over a year.

The plant has two prefiltration stages, dual media filters (sand/anthracite) which is coupled with pH adjustment to encourage flocculation and filtration cartridge units to smaller particles. The water is then forced through an array of spiral wound reverse osmosis filters to produce fresh drinking water. Due to the long lifespan and high cost of membrane units only one autopsy has been possible during this time but more frequent samples of source water, media filters and ultrafiltration units have been possible.

Sample Collection
Fresh samples were collected from cartridge filters on a number of occasions (Sept 2011, Feb 2012 and Sept 2012). Sections of the filter units were inoculated into various enrichment media including Zobell's marine broth (Zobell and Allen 1935) for isolation of environmental strains of bacteria and the remaining samples stored at -20^{o}C. Freeze-thaw increased DNA yield (personal observation). For DNA extraction, samples were thawed and small pieces cut using sterilised scissors, triplicate extractions of 0.5g wet weight were completed and DNA extracted as below. In September 2011 a whole RO unit was available for autopsy and fresh samples were again enriched in media and sections cut with sterile scissors (0.5 g) in triplicate for DNA extraction. Sand/Anthracite filter material was also collected in Sept 2012 after a breakthrough event and 0.5g aliquots used for DNA extractions in triplicate. Seawater was collected but insufficient bacterial DNA was present in small volumes (personal observation), so larger volumes (3L) were concentrated by vacuum filtration through 0.2µM polycarbonate filters. Each filter was then cut into smaller pieces for DNA extraction using the method below.

DNA Extraction
DNA was extracted using a MoBio (Solana Beach, CA) Powersoil DNA kit which incorporates a bea-beating mechanical lysis step, followed by precipitation and purification steps. The manufacturer's procedures were followed with final DNA elution carried out in 30 µl DNAse free water.

Barcoded pyroseqencing and analysis
DNA extracted was used to amplify variable regions of the bacterial 16S rRNA gene by barcoded pyrosequencing as previously described (Coghlan et al, 2012). Briefly, universal bacterial fusion primers (Hamady et al, 2008) were used to generate PCR amplicons in triplicate and pooled. PCR was carried out in a 25 µL total volume including 4 µL of template DNA, containing: 2.5 mM $MgCl_2$ (Fisher Biotec, Aus), 1× Taq polymerase buffer (Fisher Biotec, Australia), 0.4 µM dNTPs (Astral Scientific, Australia), 0.4 mg BSA (Fisher Biotec, Australia), 0.4 µM of each primer, and 0.25 µL of AmpliTaq Gold DNA polymerase (ABI). The PCR conditions included: initial denaturation at 95°C for 5 minutes, followed by 40 cycles of 95°C for 30 s, 54°C 30 s, 72°C for 30 s, and a final extension at 72°C for 10 minutes (Corbett Research, NSW, Aus). Amplicons were purified (AMpure beads, Invitrogen) and DNA concentration estimated by ethidium gel staining to approximate equimolar concentrations for emulsion PCR. Bead:template rations for the emulsion were determined by qPCR (Bunce et al., 2012). The Roche GS Junior run set up included an emulsion PCR step, bead recovery, and the sequencing run. All of these procedures were carried out according to the Roche GS Junior protocols (http://www.454.com). The sequencing output files were processed as previously described (Coghlan et al, 2012) through an automated pipeline in an Internet-based bioinformatics workflow environment, YABI (https://ccg.murdoch.edu.au/yabi/). The resultant BLAST files were imported into the proGram-MEtaGenome ANalyzer (MEGAN version 4.62.1) (Huson et al., 2007) for taxonomy using the following lowest common ancestor parameters: min score of 65, top percent of 5, and min support of 1. To compare the MEGAN (Blastn Altschul et al., 1990) assignments with other distance-based algorithms, QIIME (Caporaso et al, 2010) analysis was also conducted.

Bacterial isolation and identification
Once single colonies were obtained on enrichment media, several methods were used to obtain the most accurate identification:
i) Biochemical test kits (Biolog Gen III, Biolog Inc., Hayward, CA) according to the manufacturer's instructions which analyse the microbe based on their carbon source utilisation and chemical sensitivity. A phenotypic fingerprint of the bacteria is used for species level identification. This kit is most suited to environmental species of bacteria (Kwon et al. 2002).
ii) Matrix assisted laser desorption/ionisation (MALDI)-time of flight (TOF)/ Mass spectrometry (MS) analysis is done to identify the species based on their protein profiles. The methods described by Cherkaoui et al. (2010) were followed to analyse the protein composition of a bacterial cells by measuring the exact sizes of peptides and small proteins, specific to each bacterial species. The advantages of this method are its speed, low sample volume requirements and modest reagent costs with 99.1% accuracy.
iii) Molecular characterisation by 16s DNA gene sequencing of pure isolates was also carried out where other methods gave contraindications or no identification. DNA was extracted as above from 0.5ml dense cultures and PCR amplified using the universal bacterial primers 27F and 907R (Lane et al., 1991) with an

annealing temperature of 55^{o}C. BigDye Terminator v3.1 labelling kit was used according to the manufacturer's instructions (Applied Biosystems, Foster City, CA).

Results and Discussion

In this study, variable regions of the bacterial 16S rRNA gene were amplified by barcoded pyrosequencing as outlined above. Over 10,000 high quality 16S rDNA sequence reads were generated which included source seawater, dual media filters, cartridge filters and reverse osmosis membranes. Sampling of RO membrane units can be difficult as they have a long lifespan and are contained in sealed units. Samples from other locations which are more accessible and/or frequently replaced could be used to indicate system fouling or side stream biofilm development units may be suitable alternatives. To explore the dataset, MEGAN was used which allows information on both taxonomy and number of reads assigned to be visualised in a simple user-friendly interface (Huson et al 2007) (Figure 1 and 2). As expected, the most similar communities were evident on replicate samples from the same location, for example the three cartridge filters collected over a year (Figure 1). It appears from comparisons of cartridge filters over time and seasons that temporal effect is more significant than seasonal effects (Figure 1). Pseudomonas spp., members of the Rhizobiales family and different groups of gammaproteobacteria were present in all cartridge filter samples. In samples collected in September and February 2012 there were more species belonging to the Bacteroidetes hylum such as *Sphingomonas*, *Rhodobacteriaceae*, Desulfobacteria and Chromatiales than in September 2011 (Figure 1). The cartridge collected in September 2011 had more *Bacillus*, *Microbacteriaceae*, *Burkholderia*, *Enterobacteriaceae* and *Xanthomonadaceae* (Figure 1). It could be argued that there were more overlaps between bacterial communities on the cartridge filter collected in September 2011 and the RO membrane, collected at the same time. The cartridge filters consisted of a large number of Gammaproteobacteria, in particular of the family *Enterobacteriaceae* and also many betaproteobacteria and firmicutes such as *Bacillus* spp. The prevalence of *Bacillus* in cartridge filters was also recorded by Chun et al. 2012) and also evident in our isolated species (Table 1).

There were similarities between bacterial communities in seawater and both dual media filters and cartridge filters but there was less overlap between cartridge filters and RO membrane communities (Figure 2). This supports other studies which suggest cartridge filters are not the primary source of downstream biofilm formation, but rather the biofilms on reverse osmosis membranes are a distinct population (Zhang et al., 2011; Bereschenko et al., 2010; Chun et al., 2012). Differences in membrane characteristics may contribute to these differences (Chun et al., 2012). It appears that some bacteria are able to traverse microfiltration membranes (Wang et al., 2008) particularly slender and flexible species. These types of bacteria are commonly found in oligotrophic marine environments. There were also significant numbers of small *Microbacteriaceae* evident on cartridge filters (Figure 2). Rarefaction plots indicated adequate coverage of the bacteria was obtained with the exception of the RO membrane sample which was undersampled (data not shown). This highlights the difficulties in availability of membrane samples and DNA extraction methods on these samples.

Dominant bacterial biofilm species identified in seawater systems vary between studies. Many studies indicate the prevalence of Alphaproteobacteria in seawater (Joint et al., 2010) and RO membranes (Manes et al., 2011) including species of the *Rhodobacteriaceae* family (Sibani et al., 2007), *Roseobacter* and *Sphingomonas* spp. which have been identified as primary colonisers in several studies (Dang and Lovell, 2000; Bereschenko et al., 2010; Lee et al., 2009). On reverse osmosis membranes a larger proportion of Bacteroidetes (27%), Planctomycetes (14%) and Chloroflexi (6%) were indicated in a conventional sequencing study by Chun et al. (2012). Similar trends were evident in this study with 13% of RO membrane sequences matching the Bacteroidetes group (Flavobacteriaceae eg. Pseudoalteromonas), 3% Planctomycetes and 6% Chloroflexi (Figure 2). Another study has also highlighted the prevalence of Alphaproteobacteria such as *Sphingomonas* spp, *Bacteroidetes* and *Planctomycete* in seawater RO systems (Lee et al., 2009).

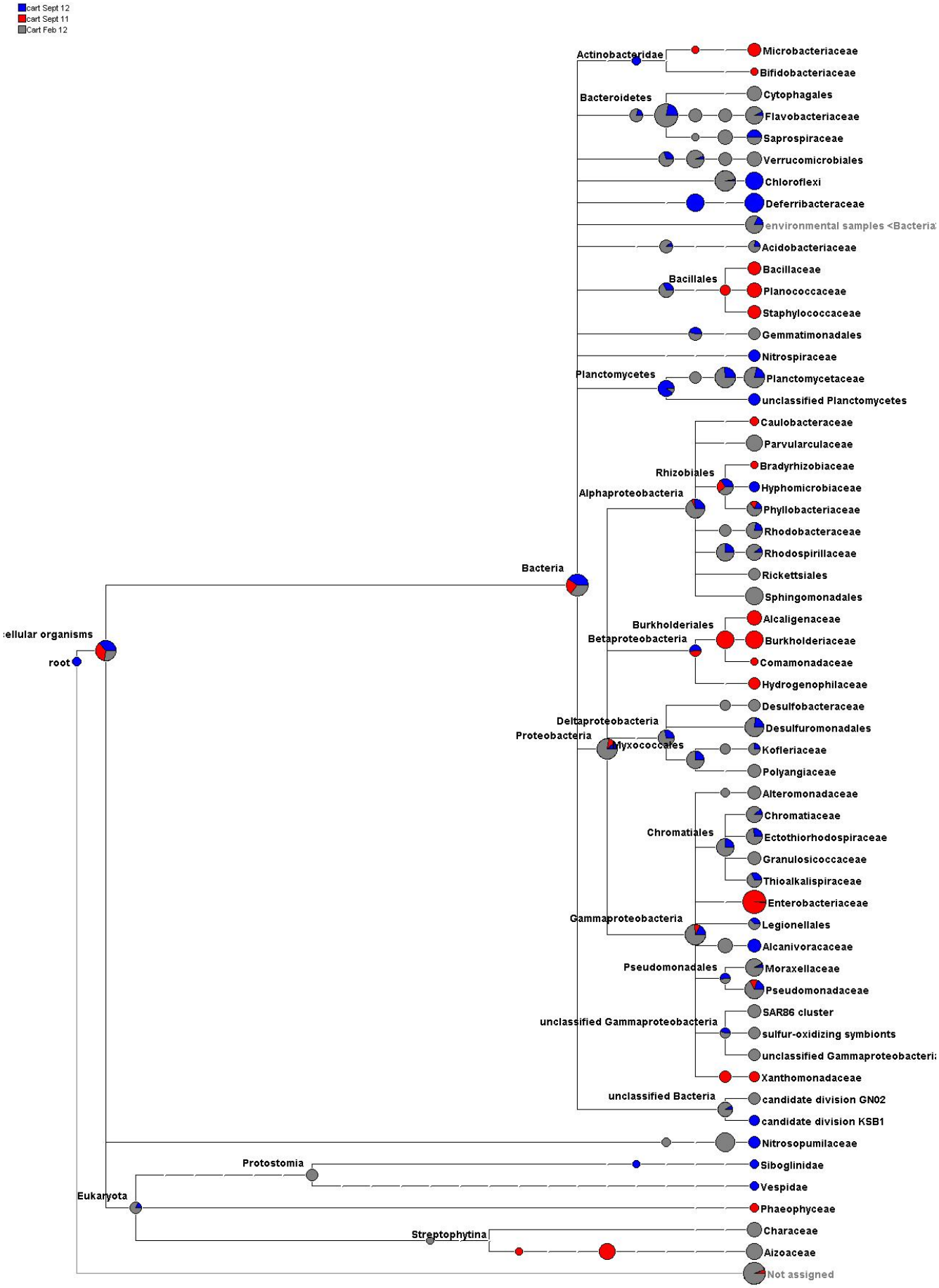

Figure 1: MEGAN phylogram of bacterial communities in cartridge filters collected in Spring (September 2011 and 2012) and summer (February 2012) to investigate seasonal and temporal changes. Size of nodes indicate relative abundance of sequence reads.

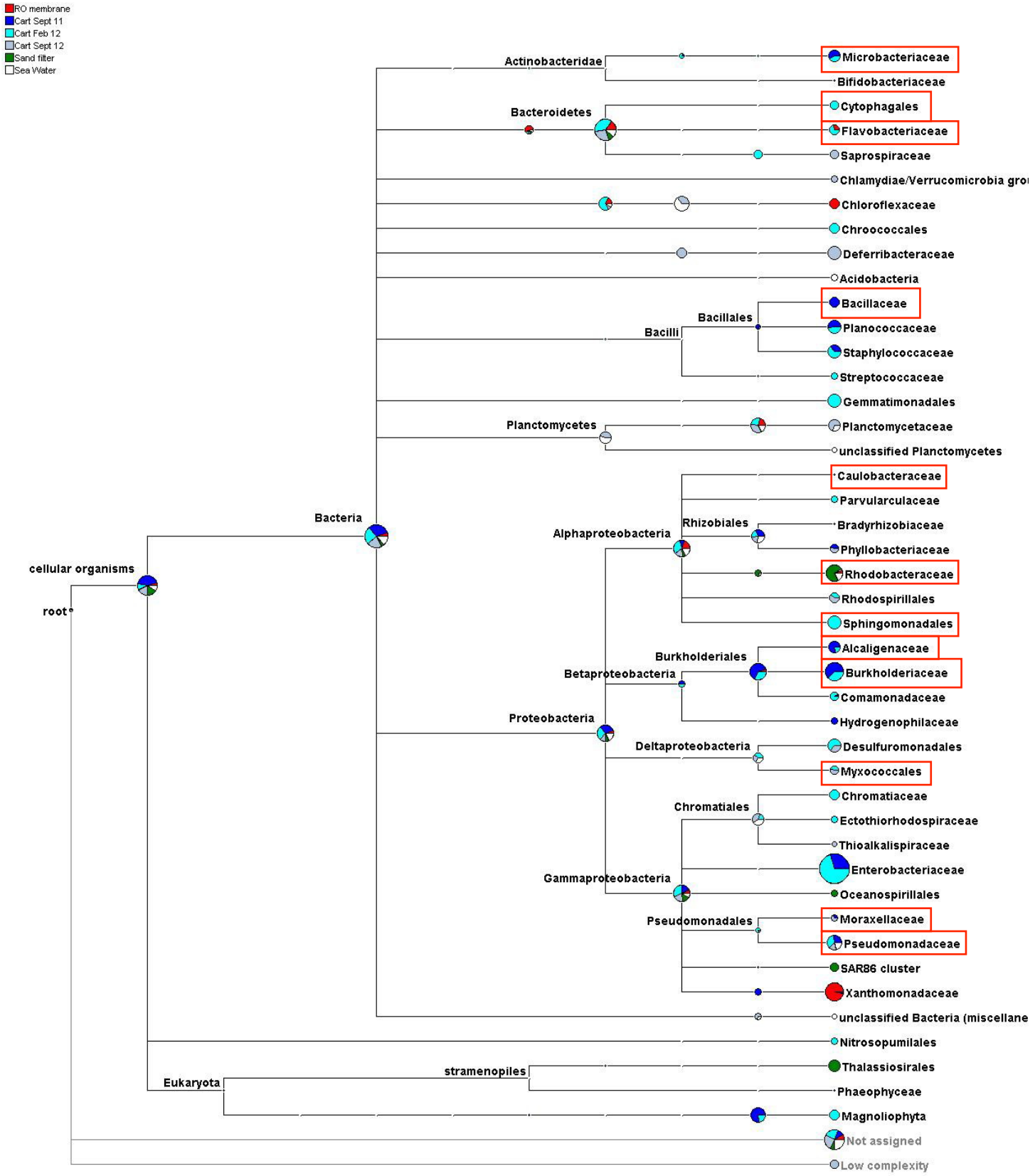

Figure 2: MEGAN phylogram of bacterial communities in samples from a full scale desalination plant in Western Australia. Colours of pie charts indicate sample date and location and size of nodes indicate relative abundance of sequence reads. Red boxes indicate environmental strains isolated and identified for further biofilm studies.

Phylum/Class/Order	Family	Seawater	Media Filter	Filtered seawater	Cartridge filter	RO membrane	Polished seawater
Actinobacteridae	*Microbacteriaceae*	1 G				1 M	
Bacteroidetes	*Flavobacteriaceae* *Cytophagales*				1 16S 1 R		
Firmicutes	*Bacillaceae*		3 M/16S		3 M	3 M	
Alphaproteobacteria	*Caulobacteriaceae* *Rhodobacteraceae* *Sphingomonadales*	1 R	1 R		1 R 1 R	3 R 1 R	1 R
Betaproteobacteria	*Burkholderiaceae* *Alcaligenaceae*	4 R		2 R	6 R	2 R 2 R	2 R 1 R
Deltaproteobacteria	*Myxococcales*			1 G			
Gammaproteobacteria Alteromonadales Pseudomonadales	 *Shewellaceae* *Pseudomonadaceae* *Moraxellaceae*	 1 G 2 G/R	1 16S	 1 R 1 R	 1 R	 8 R/M 1 R	

Table 1: Environmental isolates from different location within a full scale desalination plant indicating number of isolates and method of identification (R Remel RapID NF Plus; G Microlog GenIII; M MALDI-TOF; 16S sequencing.

We found similarities between bacterial families across sampling locations including seawater, media filters, microfiltration units and membranes but the proportions or presence of particular species differed with location, suggesting a seawater source initially followed by adaptation of the bacterial species to particular niches. Each location has particular characteristics which select bacterial groups which grow well in that niche but equally some bacterial groups appear common across all locations. This may also explain why dominant fouling species differ between studies with *Sphingomonas* spp (Lee et al., 2009), a filamentous, EPS producing bacterium, *Leucothrix mucor*, is the predominant species present in biofilms on RO membranes in California as well as marine *Aeromonas* and *Alteromonas* spp. (Jiang, personal communication) and in this study, *Xanthomonas* spp. dominating the biofilm membrane community.

Despite the dominance of different species in different studies, similarities between families of bacteria were evident in disparate locations and seasons. This would support the selection of suitable target groups for the development of rapid, molecular detection methods at the family or genus level for common fouling groups which we recommend should include members of the Bacteroidetes (eg. *Flavobacteriaceae*), Planctomycetes, Alphaproteobacteria (eg. *Rhodobacteraceae*, *Sphingomonadales*), Betaproteobacteria (eg. *Burkholderia*) and Gammaproteobacteria (eg. *Oceanospirillales*, *Xanthomonadaceae*). Family level detection would allow for variations in dominant species between locations.

Species identification of cultured isolates usually relies on phenotypic methods such as biochemical reactions, antibiotic resistance and fatty acid patterns. However, these methods have limitations such as highly related species cannot be phenotypically differentiated and corresponding databases are often limited, hampering accurate identification. To overcome these drawbacks genotypic identification methods have been widely used, and most of them are based on the polymorphism of 16S rRNA genes. Nowadays sequencing of the 16S rRNA is accepted as the reference method for species identification and several studies have shown its superiority to phenotypic methods (Mellmann et al, 2008). However a prerequisite for retrieval of valid identification of results is the use of an extensive and comprehensive quality-controlled database. Matrix assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI- TOF MS) can also be used to analyse the protein composition of a bacterial cell, has emerged as a new technology for species identification. The limitations of this method are reproducibility of results using cultivation conditions and the limited availability of reference data sets (Mellmann et al, 2008). All three methods were used as required to identify over 50 environmental isolates from the full scale desalination plant (Table 1). Like the community profiling, the culture studies also showed differences in groups isolated from RO membranes compared to cartridge filters and other locations (Table 1). Comparatively more *Bacillus* spp. were isolated from cartridge and media filters for example (Table 1). However, culture biases cannot be ruled out and it is important to compare with community profiles to select suitable model organisms for biofilm studies.

The information we now have will enable us to select a range of environmental isolates from our culture collection to model biofilms *in situ* and therefore measure the effectiveness of fouling alleviation approaches more accurately.

Conclusions

- Samples from other locations do not appear to accurately represent the communities on RO membranes, therefore sampling of RO membranes is necessary and/or use of side streams to develop biofilms.
- There are some similar species evident across locations in a full-scale desalination plant, different species appear to exploit particular niches.
- Family level molecular detection of several groups could be useful and should include Bacteroidetes (eg. *Flavobacteriaceae*), Planctomycetes, Alphaproteobacteria (eg. *Rhodobacteraceae*, *Sphingomonadales*), Betaproteobacteria (eg. *Burkholderia*) and Gammaproteobacteria (eg. *Oceanospirillales*, *Xanthomonadaceae*).
- We have selected environmental isolates from the above families as model bacteria to accurately test the effectiveness of anti-fouling strategies on RO membrane biofilms.

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